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AN INVESTIGATION INTO THE EFFECTS OF VARIOUS DILUENTS ON BACILLUS COLI COMMUNIS.

THESIS.

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AN INVESTIGATION INTO THE EFFECTS OF VARIOUS DILUENTS ON BACILLUS COLI COMMUNIS.

The following investigation was suggested by certain inconsistences observed in the course of some quantitative examinations of bacteria during which the possibility of serious errors arising in connection with dilution such as is frequently undertaken in the enumeration of viable bacteria was noted.

It appears from the literature on the subject that many authors have assumed that, provided the time-interval of dilution is short, the lethal effect of a diluent such as saline, tap-water and even distilled water can be ignored. That this is not the case is proved by the experiments recorded in this paper.

It will be shewn that in the case of B. coli and other organisms of this group the death-rate may be extremely rapid, for it is determined not only by the nature of the diluent, but also to a very marked degree by the physical state of the individual bacterial cells. Vegetative forms of B. coli communis removed from their culture medium during the logarithmic phase of growth are extremely sensitive to all of the above mentioned diluents, whereas an old culture of the same organism is much less so.

Between these extremes the bacilli shew a graduation is susceptibility. Differences in degree of staining serve to distinguish the old from the young bacteria.

The organism chosen for this work was B. coli, originally obtained from the National Collection of Type Cultures, and since maintained on beef extract agar.

The difference in staining reactions of a young and

of an old bacterial culture is a matter of daily observation in the laboratory, for while the young culture takes the stain intensely the old culture appears by contrast to be almost unstainable. That these differences are associated with a change in the viability of the organism is proved by the experiments recorded below.

It is almost certain that on ordinary laboratory media cessation of growth of a bacterial culture is due to the inability of the organism to utilise the nutriment rather than to a lack of nutriment itself. This condition is brought about by an accumulation in the immediate neighbourhood of the bacillus of the toxic products of its own metabolism. The response of the organism to its increasingly toxic environment is a profound change in its ectoplasm in consequence of which all protoplasmic activity apparently becomes stationary. The membranic envelope of the organism seemingly undergoes some change which enables the organism to withstand, for a time, the influences tending to destroy its vitality. In other words, the organism changes from the vegetative to the resistant or dormant condition.

The action of a diluent which is not in itself chemically lethal will serve by decreasing the concentration of toxin to excite the organism to begin another phase of growth.

When a diluent, such as distilled water or physiological saline, contains nothing of nutrient value the resting bacilli will first vegetate and then die out, the death of the bacilli being due probably to plasmolysis and autolysis.

The following experiments illustrate the above statement. Five cultures of B. coli were taken and studied - a 30 day old, a 16 day old, a 7 day old, a 4 day old and a 24 hour old culture. At all stages of growth smear

preparations were made and stained by Loeffler's Methylene blue. Taking the 30 day old culture, for instance, the first smear shewed that all the organisms by their very feeble staining reaction and indistinct morphology were in the resting condition. A subinoculation on to a fresh agar slope was made. The minimum amount of growth was taken to ensure viability. Smears were made at the time of each subinoculation to permit of careful observation of each stage of growth. After a short period of incubation at 37°C and as soon as a definite film of growth was detected a small portion of the culture was seeded on to a fresh agar slope. This process was repeated a third time and films were then prepared of the rapidly growing young organisms. All the bacilli shewed an intense staining reaction.

The culture was now returned to the incubator, and at intervals of ten minutes, covering a period of two hours, smear preparations were made and stained as before. The gradual change from vegetative into resting stage was clearly demonstrated. At the end of 24 hours only a few vegetative bacilli were seen, and a final preparation after four days growth shewed nothing but resting forms of the bacillus.

Another series of observations was made beginning with the growth on a slope subinoculated from an old culture. The smear made at the time of inoculation shewed only resting forms all feebly and uniformly stained. After about 40 minutes a few vegetative forms were seen together with many transition forms, i.e. forms with staining properties intermediate between the vegetative and the dormant. The vegetative forms now increased rapidly in numbers and at the end of an hour constituted practically all the bacilli. Shortly after this the vegetative phase

began to decline and a return to the dormant stage was practically complete in 24 hours.

Next, a heavy suspension of B. coli in physiological saline from a 9 day old slope culture was made and immediately centrifuged and the supernatant fluid poured away. The bacillary residue was washed twice with sterile saline. Examination of the moist deposit shewed all the bacilli were in the resting condition. A very small quantity of fresh saline was now added and the bacterial deposit placed in the incubator at 37°C and examined at intervals. Within half an hour many deeply staining bacilli were noted. These gradually increased up to a certain point, and then after a somewhat prolonged interval (over-night) disappeared. Addition of a further small quantity of saline induced another effort on the part of the bacilli to begin a fresh vegetative phase which died out as before in a few hours. Therefore to change from resting to vegetative forms the organisms evidently require to be freed from the associated inhibitory substances, i.e. the products of metabolism. The addition of a small quantity of saline partially achieves this by lowering the concentration of toxins. The survival of the vegetative forms for some hours is probably due to their being able to derive nutriment from the dead forms, for judging by microscopical observation the growth is local rather than general as would be expected if the growth were due to a trace of culture medium being carried over with the bacteria.

The rapidity with which by appropriate treatment the above changes in the bacilli can be induced to occur proves how extremely sensitive the organisms are to a change in their environment.

The vegetative forms which are so easily produced by the action of a little saline are just as effectively

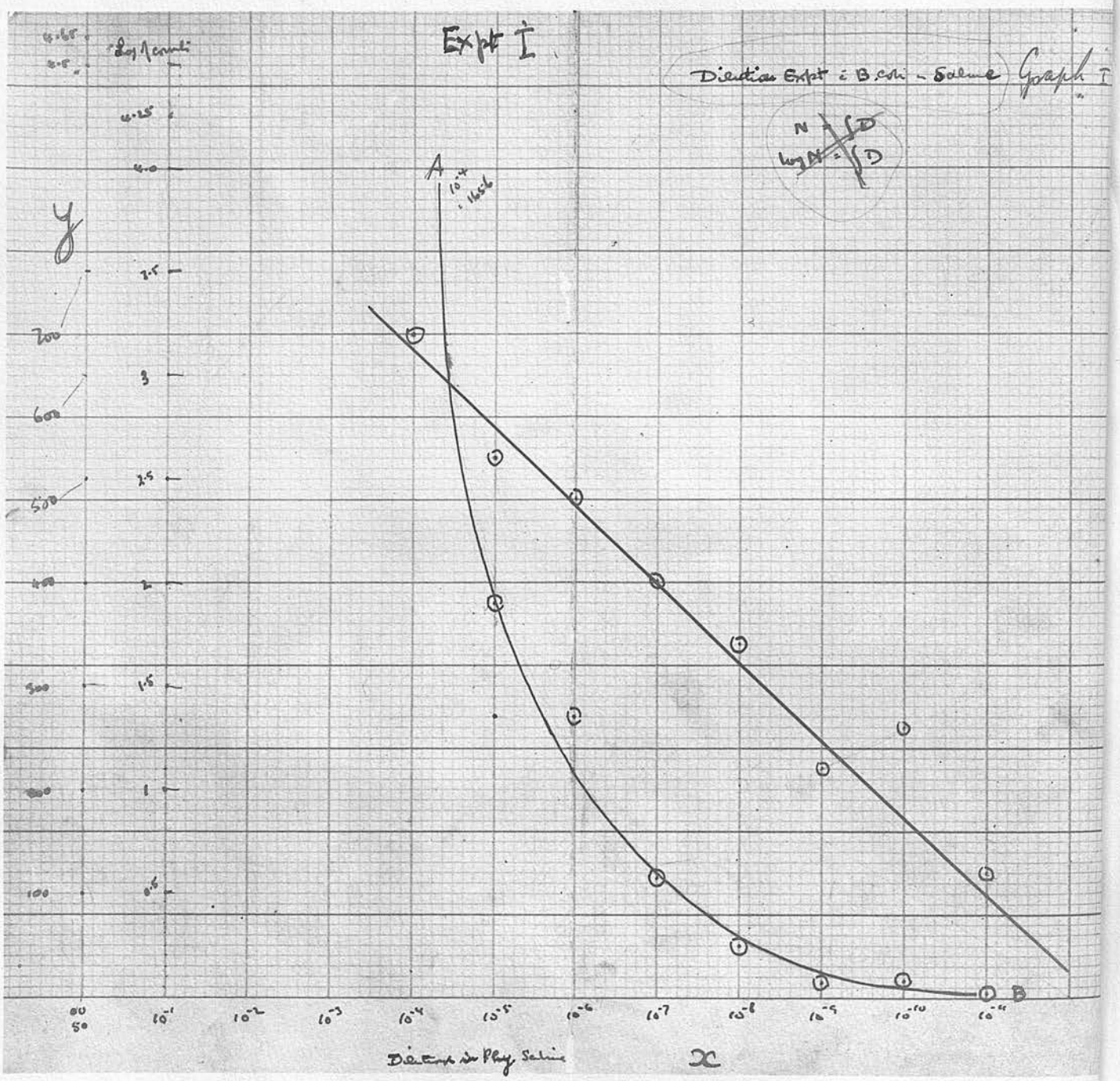
destroyed by the same agent if no fresh nutritive substance is added. This is shewn by the experiments above mentioned. From this it may be inferred that by treatment with saline or distilled water the death-rate of the vegetative and of the dormant bacilli will shew marked differences. The death-rate of the vegetative forms will probably be almost immediate whilst that of the resting stage will be more prolonged, for the organism has first to change to the vegetative form before death takes place. That these conclusions drawn from the foregoing microscopical examinations are correct is proved by a series of experiments given below in which the vegetative and the dormant forms were separately submitted to the action of saline, and of distilled water.

EXPERIMENT I.

The purpose of this experiment was to determine the magnitude of the error which can arise in estimating the number of viable bacteria in a suspension when the method adopted is that of diluting the suspension with physiological saline, making plate counts of the organisms present in the dilution, and then assuming that this number multiplied by the dilution factor represents the actual number of viable bacteria in the original suspension.

An initial suspension was prepared in saline (0.85% NaCl) of organisms from a 24 hour agar slope culture of B. coli and a series of dilutions was made as shewn.

Plates were inoculated with as little delay as possible. Each of the inoculated plates receiving 1 cc. of the respective dilutions, the nutrient agar media being poured at a temperature of 45°C and incubated for 48 hours at 37°C and then counted with the aid of a hand lens magnifying five times.


Expt I
Graph I
Dielectric Expt : B.Sch - Salmon
N = ∫D
log N = ∫D
A
B
y
x
Dielectric in Phys. Science

Dilution.	No. of organisms.	Log. of No. of organisms.
Initial suspension	1 uncountable	-
10^1	"	-
10^2	"	-
10^3	"	-
10^4	1,656	3.219
10^5	380	2.570
10^6	272	2.435
10^7	114	2.057
10^8	54	1.732
10^9	15	1.175
10^{10}	18	1.255
10^{11}	5	0.699

These results are interesting. With 1/10 as the serial factor of dilution the death-rate is logarithmic (see graph I), and it is clearly evident in this experiment that multiplication of any count by the factor of dilution would not give the correct number of viable organisms in the original suspension. To make such a calculation it would be necessary to use an equation of the following type:-

$$y = -ax + b$$

where y = log. of the No. of viable organisms

x = log. of the reciprocal of the dilution ratio.

a & b represent constants.

Let it be supposed that at dilutions of $1/10^6$ and $1/10^7$ the logs of the numbers of the organisms present in these dilutions are 2.4 and 2.0 respectively. Then by substitution we have

$$\text{I} \quad 2.4 = -a6 + b$$

$$\text{II} \quad 2.0 = -a7 + b$$

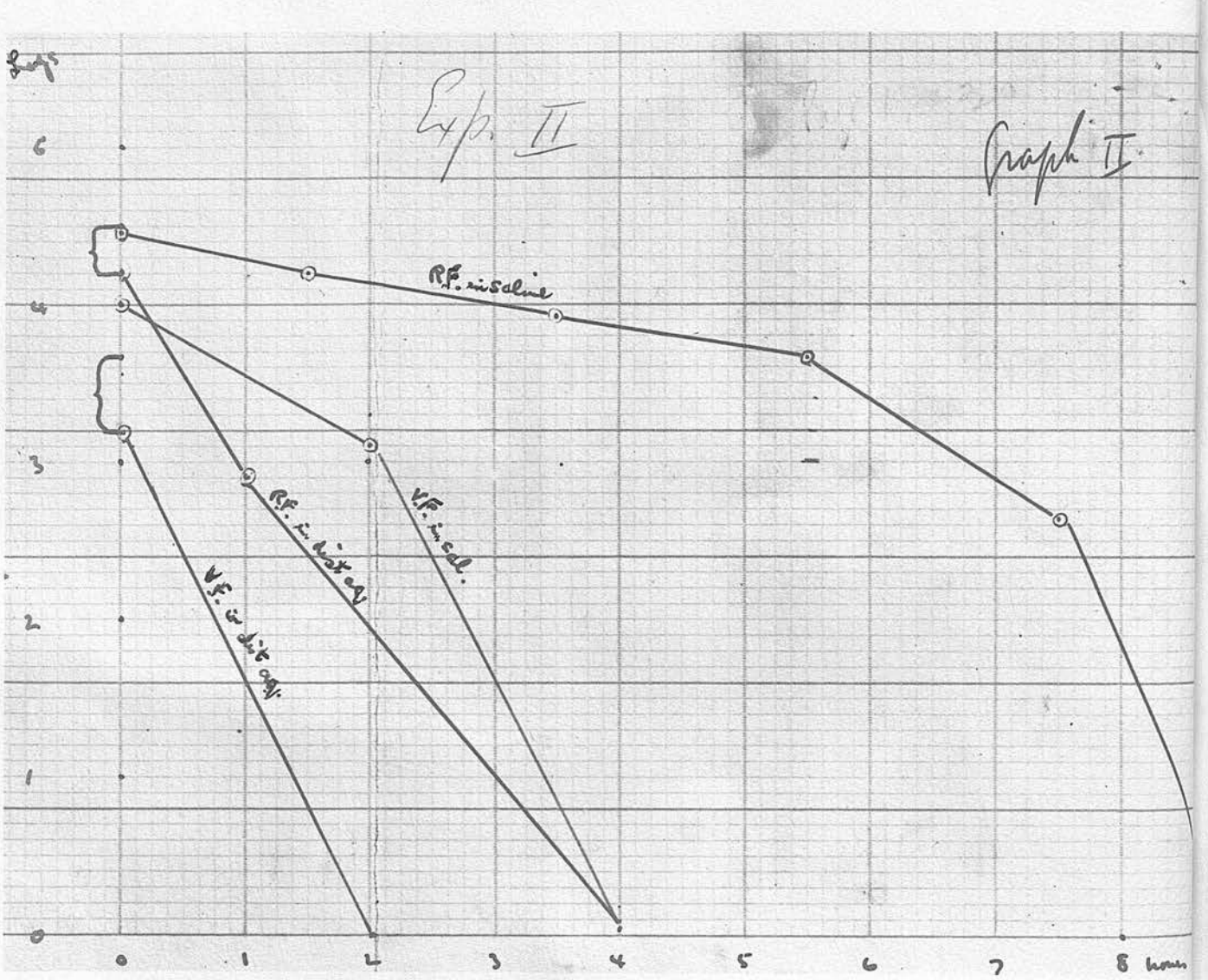
$$\text{from I} - \text{II} \therefore a = 0.4$$

$$\text{III} \quad 2.4 = (-0.4 \times 6) + b$$

$$\text{and} \therefore b = 4.8$$

Exp. II

Graph II



The original equation now becomes

$$y = -0.4x + 4.8$$

when $x = 0$, that is the value of the undiluted suspension

$$y = 4.8$$

and therefore the number of viable bacteria present in the undiluted suspension is 63,100 per cc.

EXPERIMENT II.

The purpose of this experiment was to find out the effect of dilution with physiological saline and distilled water on both the vegetative and dormant forms of B. coli.

The initial suspension was a loopful of organisms of a 24 hour old agar slope culture rubbed down in physiological saline to match an opacity of 20 opacity units.[x] This opacity is that produced by the addition to a litre of distilled water of an amount of Barium sulphate chemically equivalent to 0.02 grammes of Potassium sulphate. Of this 0.1 cc. was then pipetted into 200 cc. of saline or distilled water as the case might be. In each case where distilled water was used a control in saline was done at the same time to give some idea of the comparative number of organisms present in the original suspension. Therefore the difference between the control and the initial counts represent the number of bacteria killed off in the process of preparing the suspension in distilled water. The graphs of these figures shew very clearly the relative lethal effects.

	Physiological saline		Distilled water	
	Veg.	Dor.	Veg.	Dor.
Control counts in saline			14,800	32,000
Initial "	11,200	30,000	1,660	18,000
after 1 hour	-	-	-	800
" 1½ "	-	16,000	-	-
" 2 "	1,320	-	sterile	-
" 3½ "	-	8,900		-
" 4 "	2	-		10
" 5½ "	sterile	4,800		-
" 5⅔ "		-		sterile
" 7½ "		432		
" 24 "		sterile		

[x] A Standard Opacity Unit for Measurement of Bacterial Suspension. E.C.Haddon. Trans. Roy. Soc. Trop. Med. & Hyg. XXIII. 2.205. 1929.

Exp. II
Graph III

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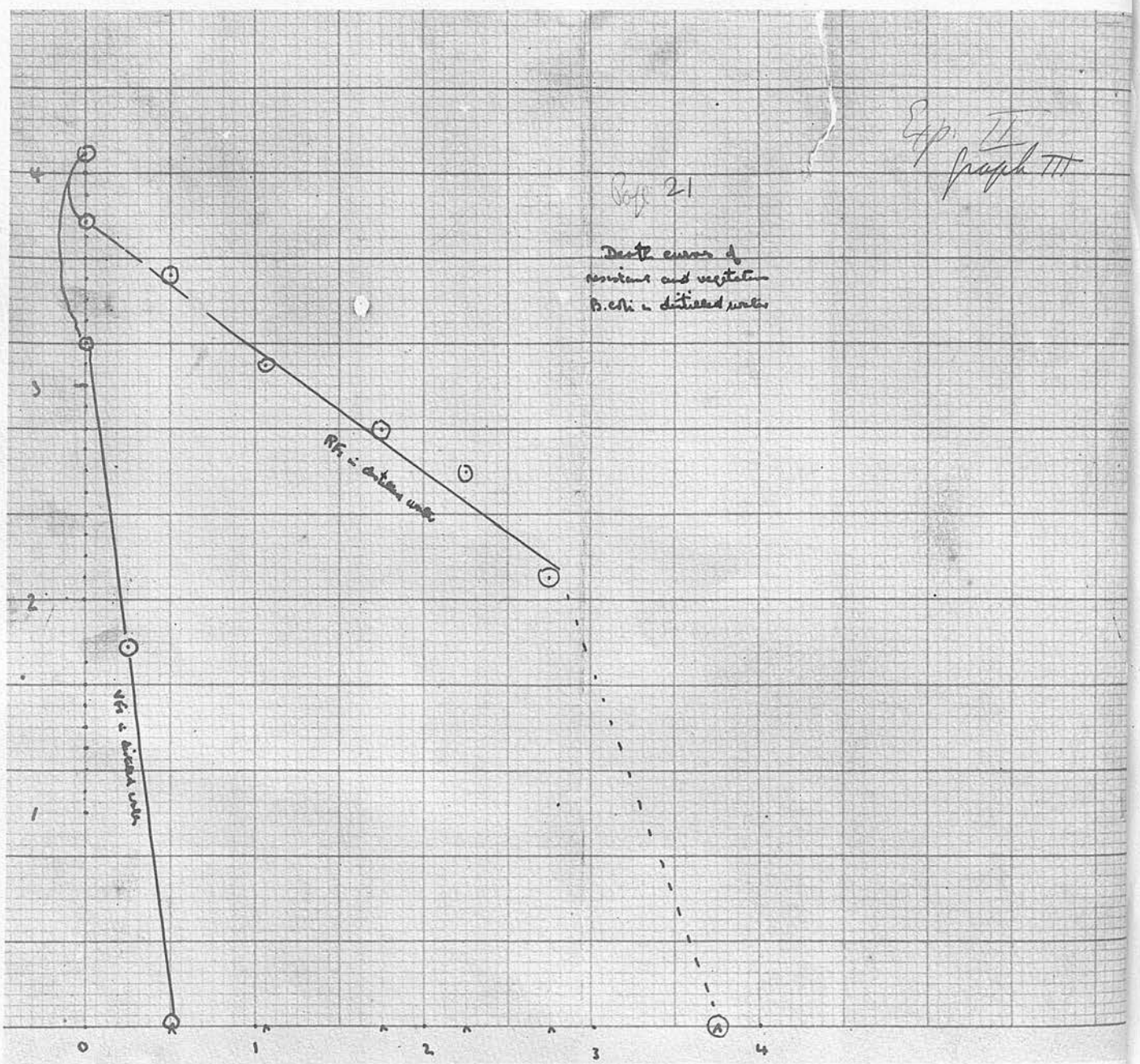


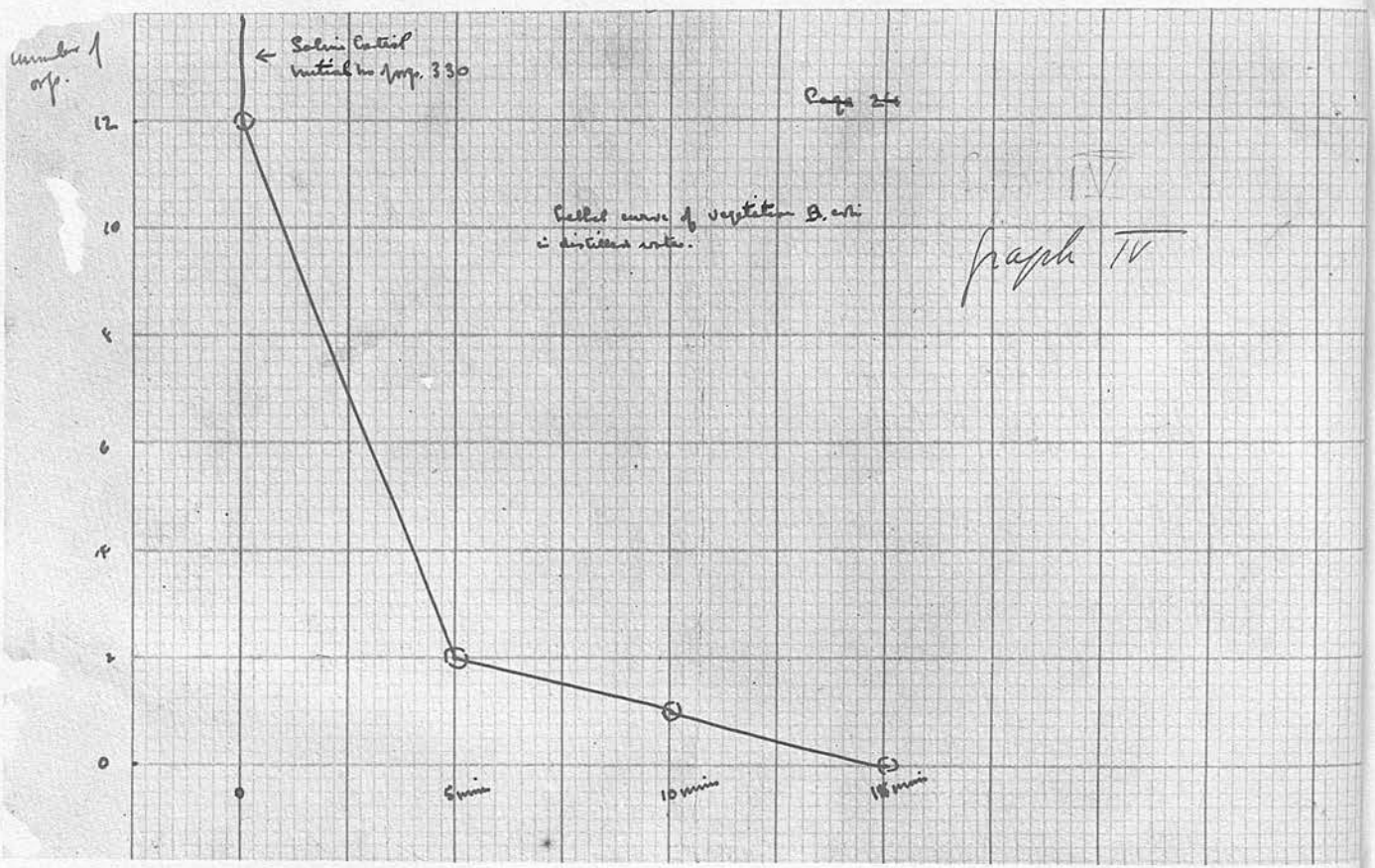
The time differences in the examinations is due to interruption of other work. It was intended that all the time intervals should be equal.

EXPERIMENT III.

The figures of the distilled water diluent proved so unexpected that a second experiment was carried out with exactly the same technique, the resulting figures and graph are shewn below.

	Veg.	Dor.
Control counts in saline	12,900	12,000
Initial counts in distilled water	1,600	6,000
After $\frac{1}{4}$ hour	60	
" $\frac{1}{2}$ "	sterile	3,400
" 1 "		1,326
" $1\frac{1}{2}$ "		689
" 2 "		408
" $2\frac{1}{2}$ "		40
" 3 "		sterile

As the death-rate of the vegetative forms in distilled water proved so rapid it seemed desirable to obtain a series of values at shorter time intervals.


Number of org.
12
10
8
6
4
2
0
0
5 min
10 min
15 min
← Solvin Control
initial no. org. 330
Page 24
Graph IV
Typical curve of vegetative B. coli
in distilled water.

EXPERIMENT IV.

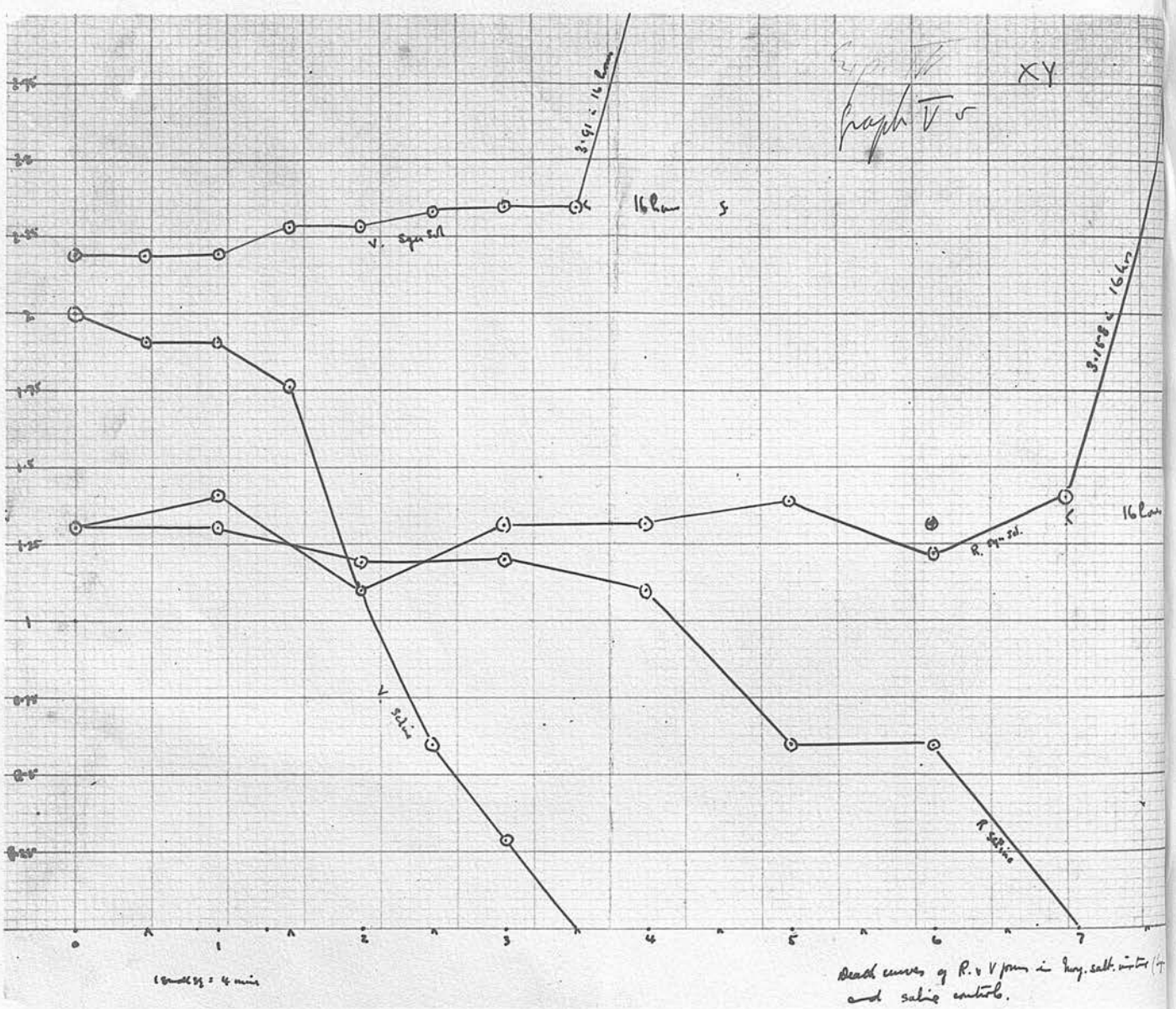
In order to obtain smaller counts the initial suspension (i.e. of 20 opacity units value) was diluted 1/100 in two stages thus: 1/10 x 1/10 and 0.1 cc. of the final dilution was added to 200 ccs. of distilled water. Theoretically this should have resulted in counts 1/100 of the original figures, i.e. 1,400 and 3,200 control instead of which the lethal effect of this additional dilution in saline was very evident. However, the numbers are sufficient for the purposes of this experiment.

Control count in saline	330.
Initial count in distilled water	12.
After 5 minutes	2.
" 10 "	1.
" 15 "	sterile.

The necessity of taking into account the lethal effects of the diluents employed in diluting suspensions of viable bacteria is proved by the foregoing experiments. It is obvious that neither physiological saline nor distilled water should be employed for this purpose even when the total time employed in diluting and plating out is short.

It is essential that a diluent should be used which will not impair the vitality of the organism, and will be equally innocuous to both the vegetative and the dormant forms, for as has been shewn both these forms may be present in a 24 hour culture of B. coli on nutrient agar.

Further dilution experiments were carried out using Ringer's solution and a solution of mixed inorganic salts. The composition of the two solutions being:-



(Squares = 4 min

Death curves of R. & V. spp. in hyp. salt. water (4
and saline controls.

Ringer's Solution.

Sodium chloride	0.80%
Calcium chloride	0.02%
Potassium chloride	0.02%

Mixed Inorganic Salt Solution.

Potassium phosphate (K_2HPO_4)	0.5 grammes
Magnesium sulphate	0.5 "
Ammonium sulphate	0.5 "
Calcium chloride	0.2 "
Sodium nitrate	0.5 "
Distilled water	1,000 ccs.

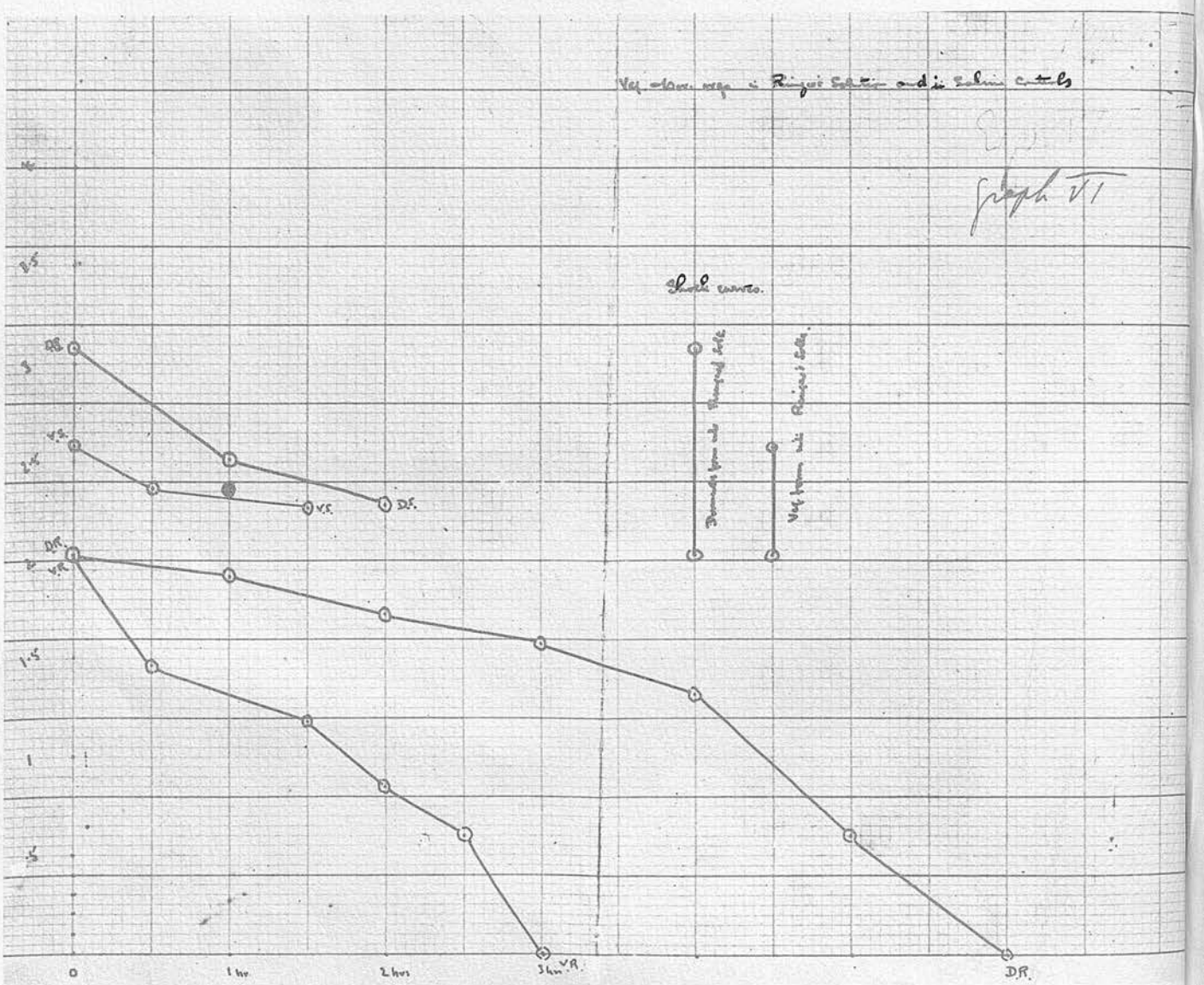
It was known that the latter medium was capable of supporting the life of B. coli but the rate of growth promised to be practically negligible within the time required for diluting and plating out of bacterial suspensions.

EXPERIMENT V.

In this experiment the effects of physiological saline and the mixed salt solution on both vegetative and dormant B. coli communis were determined. The results tabulated and graphed are given below:-

	Physiological saline.		Mixed salt solution.	
	Veg.	Dor.	Veg.	Dor.
Initial counts	114	20	160	20
After ½ hour	80	-	166	-
" 1 "	82	20	168	26
" 1½ "	51	-	186	-
" 2 "	14	15	185	14
" 2½ "	4	-	224	-
" 3 "	2	16	230	21
" 3½ "	1	-	230	-
" 4 "	Sterile	12	-	22
" 4½ "		-		-
" 5 "		4		24
" 6 "		4		21
" 7 "		3		22
" 8 "		sterile		21
" 24 "		sterile	8200	1440

The graph speaks for itself. The difference between the physiological saline and mixed salt solution is well marked particularly in regard to the respective actions on the vegetative bacilli.


Graph VI
Short course.
Average from the Municipal Office
Vaccine from the Pasteur Institute
DS.
VS.
DR.
VR.
0
1 hr.
2 hrs
3 hrs
VR.
DR.

EXPERIMENT VI.

	Physiological saline.		Ringer's solution.	
	Veg.	Dor.	Veg.	Dor.
Initial counts	385	1,200	103	102
After ½ hour	237		29	
" 1 "	190	320	15	83
" 1½ "			7	
" 2 "		193	4	54
" 2½ "			sterile	
" 3 "				38
" 3½ "				
" 4 "				21
" 4½ "				
" 5 "				4
" 5½ "				
" 6 "				sterile

The above figures and graph shew the lethal effects of Ringer's solution which is practically as inimical to the life of the bacilli as saline solution. The 'shock death-rate' is again a notable feature.

EXPERIMENT VII.

The marked lethal effect of water on B. coli suspended therein suggested that this principle might be usefully applied in determining the relative lethal action of natural waters. To test this idea five samples of water from different sources were collected and 100 cc. of each sample placed in plugged flasks and sterilised, together with similar controls of distilled water and saline.

Each flask was now inoculated with one loopful of a suspension of B. coli (of 50 opacity units) gently shaken and quantities of 1 cc. each plated out. After an interval of 15 minutes further 1cc. quantities were plated out. The plates were incubated at 37°C for 24 hours and the colonies then counted with the aid of the hand lens

Sample	No. of organisms. 1st count	No. of organisms. 2nd count	Relative lethal ratio. (0.85% NaCl = unity).
1.	161	9	7.3
2.	169	9	5.2
3.	143	28	2.0
4.	121	81	0.6
5.	170	54	1.3
0.85% NaCl	164	67	1.0
Distilled water	4	sterile	

The figures for the relative lethal ratio are obtained by dividing the ratios of the counts of the samples by the ratio of the counts for the 0.85% NaCl solution. Thus we have

$$\frac{161}{9} \times \frac{67}{164} = 7.3$$

SUMMARY.

1. During growth on ordinary culture media there occurs in the case of B. coli a physiological process analagous to spore formation and germination. These two conditions of the bacilli, i.e. the dormant and the vegetative can readily be distinguished by the difference of intensity of staining reaction and also by the lethal effects of physiological saline and distilled water on them.

2. The use of physiological saline and distilled water is shewn to be inadmissable for the purpose of diluting down suspensions of viable bacteria. The formula is given for a diluent which appears to be suitable for the purpose.

3. Experiments shew that the sensitiveness of B. coli to slight variations in its environment might be utilised as a biological test in the examination of water supplies.